



IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF : :

N. KIMURA ET AL. : GROUP ART UNIT: 1637

SERIAL NO.: 09/771,943 : :

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FOR: IMMOBILIZED NUCLEIC ACID AND METHOD FOR DETECTING
NUCLEIC ACID

DECLARATION UNDER 37 C.F.R. 1.132

ASSISTANT COMMISSIONER FOR PATENTS

WASHINGTON, D.C. 20231

SIR;

I, Naoki Kimura, a citizen of Japan, one of the inventors of the above-identified application, hereby declare and state that:

The following experiments were conducted by me or under my direct supervision.

Materials and methods

Synthesis of oligonucleotides

5'-Hexachlorofluorocin (HEX)-labeled and 5'-NH₂-modified oligonucleotides were synthesised on an ABI3900 DNA synthesiser at a 0.2 μmol scale using the standard phosphoramidite method. The HEX-labeled and NH₂-modified oligonucleotides were purified by reverse-phase HPLC using a standard procedure and dried *in vacuo*. Poly thymine modified- (T), poly uracil modified- (U), poly 4-thio-dT modified- (S), poly 4-thio-dU modified- (S'), poly O4-triaz-dT modified- (Z) and poly O4-triaz-dU modified-oligonucleotides (Z') were also synthesised on an ABI3900 DNA synthesiser at a 0.2 μmol scale using standard procedures. The oligonucleotides were then purified on a reverse-phase cartridge following a standard procedure and dried *in vacuo*. The sequences of oligonucleotide probes are shown in Table 1. 4-thio-dT, 4-thio-dU, O4-triaz-dT and O4-triaz-dU were purchased from Glen Research.

Synthesis of photopolymer-labeled oligonucleotides

Co-polymer (Photopolymer) consisting of acrylamide, BBA-APMA and MAL-EAC-NOS, and Photopolymer-modified oligonucleotides utilizing the 5'-NH₂-modified oligonucleotide were synthesized according to the Example 3 of U.S.

Patent No. 6,506,895. The structure of the synthesized co-polymer was confirmed by ¹H-NMR (a Joel JNM-GSX270 FT NMR system GSX FT NMR spectrometer).

Fabrication of arrays

A GT-MASS DNA microarray spotter (NIPPON LASER & ELECTRONICS LAB) was used to print arrays on polycarbodiimide-coated glass slides (dimension 75 x 25 x 1 mm) and polypropylene slides (dimension 75 x 25 x 1 mm). Either oligonucleotides in 2 M NaCl (total volume 20 μ l) or oligonucleotides in 50 mM phosphate buffer (pH 8.5) and 1 mM EDTA were used to print arrays, ~300 μ m in diameter and 500 μ m center-to-center. Oligonucleotides were printed from 96 well plates (NUNC) at the desired concentration. After printing, the arrays were UV-irradiated (254 nm, 600 mJ/cm²) for 5 min using a UV Stratalinker 2400 (Stratagene) or were incubated at 37°C for 2 hr and UV-irradiated (335 nm, 1.5 mW/cm²) for 2 min using a CRM-FA Spectro Irradiator (JASCO). The slides were treated with 3% BSA solution for 15 min at 37°C and washed with TE buffer (pH 7.2), and dried for storage.

PCR amplification

For bacteriophage lambda DNA, the PCR mix included 5 ng of phage lambda DNA (TAKARA), 20 mM Tris HCl (pH 8.0 at room temperature), 100 mM KCl, 2.5 mM MgCl₂, 5 pmol of non-fluorescent-labeled forward primer (5' tcgccccgcgttttgatga 3'), 5 pmol of non-fluorescent-labeled reverse primer (5' catcgtcgcgcggtagtcat 3'), 2.5 mM of each deoxynucleoside triphosphate, and 2.5 U of r-Taq (TAKARA) in a total volume of 50 μ l. Amplification consisted of 40 cycles, with denaturation at 95°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The initial denaturation was at 95°C for 5 min. Finally, the PCR mix was incubated at 72°C for 3 min. PCR product was purified by Qiaquick PCR purification kit (Qiagen), and cloned into the pGEM-Teasy (Promega) following the manufacturer's protocols. The plasmids were amplified in *E. coli* JM109 (Toyobo). The products were purified by a Plasmid purification kit (Qiagen).

For the generation of complementary hybridization targets, PCR reactions were performed using fluorescent-labeled forward and reverse primers with plasmid DNA as template. The PCR temperature cycle was the same as that used above. The PCR products were applied to a 5 % acrylamide gel and visualised by staining with ethidium bromide. The size of PCR product was 300 bp for bacteriophage lambda DNA. The sequences of the products were confirmed by ABI 310 genetic analyser.

Hybridization and imaging

Hybridization was carried out as follows. Fluorescently labeled PCR product (46 ng) was dissolved in 4 μ l of distilled water and denatured for 1 min at 95°C, and 16

μ l of ArrayIt (Telechem International) was added to the solution. The hybridization solution was applied by capillary action between a slide and cover slip. The slide was incubated for 2 hr at 42°C in a closed hybridization cassette. Subsequently, the arrays were washed at room temperature in 2 x SSC -0.1% SDS for 5 min, followed by 5 min in 0.2 x SSC -0.1% SDS at 40°C, rinsed briefly in 0.5 x SSC and dried by centrifugation at 500 r.p.m. (CS-15 Centrifuge, BECKMAN) for 2 min. The arrays were imaged on a Scan Array 4000 unit (Packard Biochip) with 10 μ m resolution. A Cy3 optical filter was used during imaging of the arrays. The laser power and photomultiplier tube voltage (PMT) were always set to 90% for the Cy3 channel. Analysis of the intensity of the original 16 bit tiff images from a Cy3 channel was performed using Quant Array software (Packard Biochip), and graphs were generated in Microsoft Excel. For all experiments, the average signal values were taken from the spots of 3 slides processed in parallel.

Stripping procedure

After hybridization with HEX labeled target, the slides were immersed in stripping buffer (2.5 mM Na₂HPO₄, 0.1% SDS) at 95°C for 30 s, four times (*Nucleic Acids Res.*, **27**, 1970-1977 (1999)). They were then rinsed in distilled water, dried for storage at room temperature.

Results

After an initial hybridization with a HEX labeled target, the arrayed slides were rendered to the stripping procedure and re-hybridized with the same HEX labeled target four successive times. Quantitative measurement of mean signal intensities yielded the results shown in Figure 1. Whereas the intensity of signals of probe 7 on the polycarbodiimide-coated slide and on the polypropylene slide decreased with each successive hybridization, probes 1-6 on the both slides clearly remained detectable and proportionately accurate, reflecting the amounts of specific probe DNA immobilized. Non-specific signals (probes 8-14) were not detected in both slides (not shown).

Table and Figure

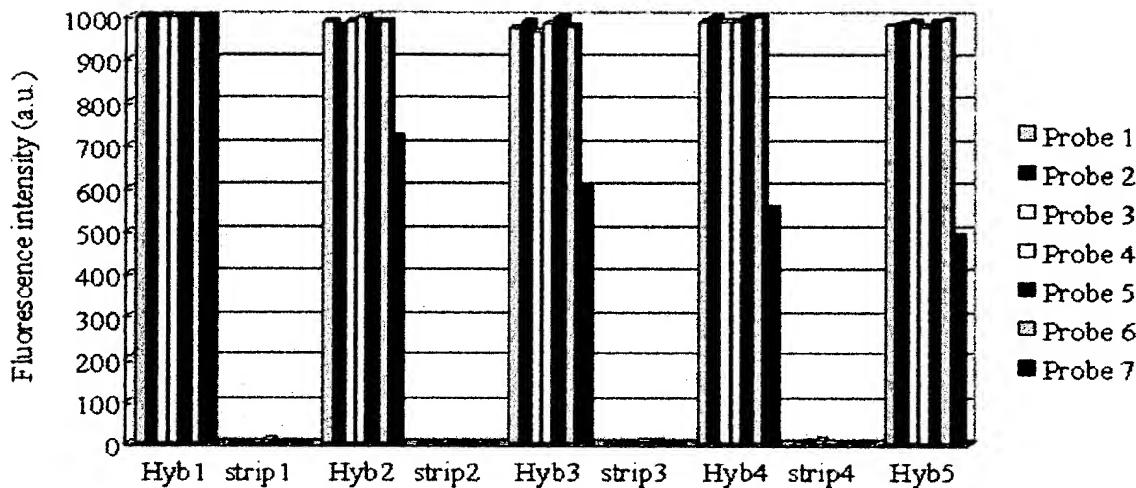
Table 1. Sequences of oligonucleotide probes

Oligonucleotide		Sequence (5'→3')	Modification	Remarks
1	Probe 1	TTTTTTTTTT tcgccccgtgttttgcata	Thymine	C
2	Probe 2	UUUUUUUUUUU tcgccccgtgttttgcata	Uracil	C
3	Probe 3	SSSSSSSSSS tcgccccgtgttttgcata	4-thio-dT	C
4	Probe 4	S'S'S'S'S'S'S'S' tcgccccgtgttttgcata	4-thio-dU	C
5	Probe 5	ZZZZZZZZZZ tcgccccgtgttttgcata	O4-triaz-dT	C
6	Probe 6	Z'Z'Z'Z'Z'Z'Z'Z' tcgccccgtgttttgcata	O4-triaz-dU	C
7	Probe 7	Photopolymer - tcgccccgtgttttgcata	Photopolymer	C
8	Probe 8	TTTTTTTTTT gttatgggtgaaactctgggag	Thymine	N
9	Probe 9	UUUUUUUUUUU gttatgggtgaaactctgggag	Uracil	N
10	Probe 10	SSSSSSSSSS gttatgggtgaaactctgggag	4-thio-dT	N
11	Probe 11	S'S'S'S'S'S'S'S' gttatgggtgaaactctgggag	4-thio-dU	N
12	Probe 12	ZZZZZZZZZZ gttatgggtgaaactctgggag	O4-triaz-dT	N
13	Probe 13	Z'Z'Z'Z'Z'Z'Z'Z'Z' gttatgggtgaaactctgggag	O4-triaz-dU	N
14	Probe 14	Photopolymer - gttatgggtgaaactctgggag	Photopolymer	N

C: Complementary strand to the target DNA

N: Non-complementary strand to the target DNA

A



B

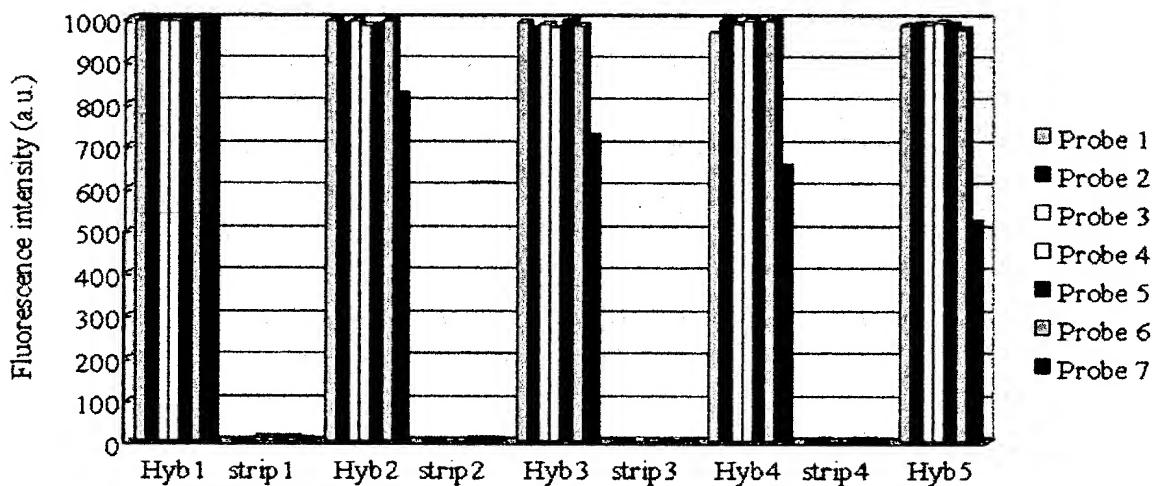


Figure 1. Effect of stripping on re-hybridization of HEX labeled target to immobilized oligonucleotides on the slides. Probes 1-6 and probes 8-13 were dissolved in NaCl buffer. Probe 7 and probe 14 were dissolved in phosphate buffer. Probes 1-6 and probes 8-13 spotted arrays were UV-irradiated (254 nm), and probe 7 and probe 14 spotted arrays were incubated at 37°C for 1.5 hr and UV-irradiated (325 nm). (A) Hybridization signals of immobilized oligonucleotides on the polycarbodiimide-coated slide and (B) on polypropylene slide surfaces. Scanned spots over hybridizations 1-5 (where 1 is the initial hybridization, and 2-5 are sequential hybridizations after repeated stripping).

I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: October 16, 2003

Naoki Kimura

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